Effect of ischemia on soluble and particulate guanylyl cyclase-mediated cGMP synthesis in cardiomyocytes

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Agullo, Luis, David Garcia-Dorado, Noelia Escalona, Marisol Ruiz-Meana, Javier Insete, and Jordi Soler-Soler. Effect of ischemia on soluble and particulate guanylyl cyclase-mediated cGMP synthesis in cardiomyocytes. Am J Physiol Heart Circ Physiol 284: H2170–H2176, 2003.—The effect of simulated ischemia [hypoxia, no glucose, extracellular pH (pH\textsubscript{e}) 6.4] on cGMP synthesis induced by stimulation of soluble (sGC) or particulate guanylyl cyclase (pGC) was investigated in adult rat cardiomyocytes. Intracellular cGMP content was measured after stimulation of sGC by S-nitroso-N-penicillamine (SNAP) or stimulation of pGC by natriuretic peptides [urodilatin (Uro), atrial natriuretic peptide (ANP), or C-type natriuretic peptide (CNP)] for 1 min in the presence of phosphodiesterase inhibitors. After 2 h of simulated ischemia, a decrease of >50% was observed in pGC-dependent cGMP synthesis, but no significant change was observed in sGC-dependent cGMP synthesis. The reduction in cGMP synthesis caused by simulated ischemia was mimicked by extracellular acidosis (pH\textsubscript{e} 6.4), which decreased pGCMediated cGMP synthesis without altering sGC-mediated cGMP synthesis. An extreme sensitivity of pGC activity to low pH was also observed in membrane cell fractions. Hypoxia without acidosis (pH\textsubscript{e} 7.4) profoundly depressed cellular ATP content but did not change the response to SNAP, Uro, or ANP (selective agonists of pGC type A receptor). Only cGMP synthesis in response to CNP (a selective agonist of pGC type B receptor) was significantly reduced by ATP depletion. These data support the relevance of intracellular pH as a modulator of cGMP and suggest that, in ischemic cardiomyocytes, synthesis of cGMP would be mainly nitric oxide dependent.

THE INTRACELLULAR MESSENGER cGMP mediates many of the most important actions of nitric oxide (NO) and atrial natriuretic peptide (ANP). NO activates cGMP synthesis by binding to a prosthetic heme group of soluble guanylyl cyclase (sGC), whereas ANP and ANP-related peptides stimulate particulate, membrane-bound guanylyl cyclases (pGCs).

cGMP has been shown to reduce vascular permeability (8, 15), cell attachment to the endothelial wall (7, 21), and myocardial reperfusion injury (2, 11). Recently, it has also been implicated in late preconditioning (14). Despite the evidence that cGMP may influence cell survival during transient myocardial ischemia, little is known about the consequences of ischemia on cGMP synthesis in myocardial cells. We recently described a marked reduction of cGMP content in rat and pig myocardium after transient sublethal ischemia (11, 25). However, other authors reported increases (3, 18) or no change (20) in cGMP content in rat hearts subjected to up to 30 min of ischemia. In cultured endothelial cells from rat hearts, simulated ischemia markedly inhibited sGC- and pGC-dependent cGMP synthesis (1). Depletion of ATP and a decrease in intracellular pH were responsible for the inhibitory effect (1). To our knowledge, the effects of ischemia on cGMP synthesis in isolated cardiomyocytes have been described in only one report (6). In this study, it was found that hypoxia potentiates NO-mediated cGMP synthesis. Neither the effects of low Po\textsubscript{2} on pGC-mediated cGMP synthesis nor the influence of intracellular acidosis on sGC- and pGC-dependent cGMP synthesis has been previously reported.

The aim of this study was to characterize the effects of ischemia-reperfusion on the two pathways that regulate intracellular cGMP concentration in adult rat cardiomyocytes. Changes in cGMP synthesis mediated by sGC and pGC were investigated by analyzing the response of freshly isolated adult cardiomyocytes to the NO donor S-nitroso-N-acetylpenicillamine (SNAP) and the natriuretic factors urodilatin (Uro), ANP, and C-type natriuretic peptide (CNP). Uro is an ANP-related peptide that has been shown to reduce cardiomyocyte cell death secondary to transient ischemia in a variety of models, including the in situ pig heart (11, 25). In addition, the contributions of ATP depletion and acidosis to the inhibitory effect of ischemia on cGMP synthesis were investigated.

MATERIALS AND METHODS

The care and use of animals conformed with the guidelines of the National Institutes of Health [DHHS Publication No. (NIH) 85-23, Revised 1996], and the experimental procedures were approved by the Research Commission on Ethics of Hospital Vall d’Hebron.

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Cardiomyocyte isolation. Cardiomyocytes from the heart were obtained as previously described (2). Hearts from adult male Sprague-Dawley rats (300 g) were retrogradely perfused in a Langendorff system with a modified Krebs-Henseleit bicarbonate buffer (in mM: 110 NaCl, 2.6 KCl, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 25 NaHCO$_3$, and 11 glucose, pH 7.4) containing 30 mM CaCl$_2$ and 0.03% collagenase. After centrifugation (25 g for 3 min), cells from the pellet were subjected to a progressive normalization of Ca$^{2+}$ levels (up to 1 mM). Rod-shaped cells were selected by gravity sedimentation in 4% albumin gradient and plated in culture dishes containing 300 HEPES plus 4% fetal bovine serum. At the end of the procedure, culture dishes contained 85–98% quiescent rod-shaped cells.

Stimulated ischemia and reperfusion. Cells were allocated to one of the following treatments. In normoxia, cells were incubated for 2 h in HEPES-buffered saline (in mM: 120 NaCl, 3.6 KCl, 7.4 Tris, 1 CaCl$_2$, 20 HEPES, and 5 glucose) at pH 7.4. In stimulated ischemia, cells were incubated under a 100% N$_2$ atmosphere in the same buffer but without glucose and at pH 6.4. The relative contributions of Ca$^{2+}$ dipolarosis to the decline in intracellular pH were obtained in nonstimulated cells. In additional experiments, stimulation of cGMP synthesis with Uro or SNAP in the presence of cGMP and enzyme activity was determined by radioimmunoassay, as described previously. In these conditions, the formation of cGMP was linear with time for at least 120 min. Simulated ischemia and reperfusion.

Measurement of cGMP synthesis. After different periods of exposure to the allocated treatment, cells were stimulated for 1 min (unless otherwise indicated) with 100 M SNAP, 1 M Uro, 1 M ANP, or no drug. cGMP degradation was inhibited by addition of 1 mM 3-isobutyl-1-methylxanthine (IBMX) during the stimulation period. cGMP was quantitated in cell extracts by radioimmunoassay using acetylated [3H]cGMP (2). In some experiments, a fraction of the incubation medium was collected at the end of the stimulation period to quantify the efflux of cGMP into the extracellular medium. cGMP synthesis produced by stimulation with SNAP or Uro in the different incubation conditions was expressed as a percentage of the cGMP produced after stimulation of normoxic cells (at pH 7.4) with the same drug (control plates of each batch). Before the percentage was calculated, cGMP content in nonstimulated cells (subjected to the same conditions) was subtracted from each cGMP value in the stimulated cultures. Nonstimulated cGMP was always <5% of the stimulated cGMP content.

Lactate dehydrogenase release, ATP content, and intracellular pH. Lactate dehydrogenase (LDH) activity was spectrophotometrically measured in the incubation media (2) and expressed as percentage of the total LDH content determined in the cultures after homogenization in Tris-HCl buffer. LDH content was measured in cell cultures immediately frozen in liquid nitrogen by means of the Bioluminescent Somatic Cell Assay Kit (1). Changes in intracellular pH were analyzed using a ratio-fluorescence imaging system (QuantiCell 2000, Visitech), as previously described (1). After cardiomycocytes were loaded with 1 M 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) acetoxyethyl ester, sequential 450 nm-to-490 nm ratio images were obtained during the 120 min of exposure to the different treatments. Calibration of the BCECF ratio signal was performed in the presence of nigericin (10 µg/ml). All data on ATP content and intracellular pH were obtained in nonstimulated cells. In additional experiments, stimulation of cGMP synthesis with Uro or SNAP for 2 min did not modify ATP or pH values (data not shown).

Measurement of soluble and membrane guanylyl cyclase activities. Cell cultures were homogenized with buffer A (in mM: 50 Tris-HCl (pH 7.4), 250 sucrose, 1 EDTA, and 1 dithiothreitol) plus protease inhibitors (2; the protein kinase inhibitor staurosporine (1 × 10$^{-3}$ M), and the Ser/Thr protein phosphatase inhibitors okadaic acid (1 × 10$^{-3}$ M) and cyperemphrin (5 × 10$^{-4}$ M) in a Potter-Elvehjem homogenizer. After centrifugation (100,000 g for 1 h), sGC activity was determined by incubating the soluble extract with no additions (basal) or 100 M SNAP in assay buffer (final concentrations (in mM): 50 Tris-HCl (pH 7.4), 1 EGTA, 1 dithiothreitol, 5 GTP, 4 MgCl$_2$, 15 phosphocreatine, and 1 M CNP, or no drug. cGMP degradation was inhibited by addition of 1 ml of cold ethanol, and cGMP produced by enzyme activity was determined by radioimmunoassay, as described previously. In these conditions, the formation of cGMP was linear with time for at least 30 min. In the experiments measuring pH dependence of guanyly cyclase activity, Tris-HCl was substituted for 30 M PIPEs (pH 6.0–6.8) or 30 mM HEPES (pH 6.8–7.4).

Data analysis and statistics. Statistical analysis was carried out with commercially available software (SPSS 8.0.0). Differences between groups were evaluated using one-way analysis of variance. Individual comparisons between groups were performed using the Student-Newman-Keuls test. A critical P value of 0.05 was used. Values are means ± SE. Materials. Uro was kindly provided by Prof. Dr. Wolf-Georg Forssmann and Dr. Markus Meyer (Niedersächsisches Institut für Peptid-Forschung, Hannover, Germany). SNAP, ANP, CNP, DNP, IBMX, Tris, HEPES, PIPES, GTP, staurosporine, and the Bioluminescent Somatic Cell Assay Kit were obtained from Sigma, cyperemphrin and okadaic acid from Calbiochem, [3H]cGMP (35 Ci/mmol) from New England Nuclear, collagenase from Serva, BCECF from Molecular Probes, plastic petri dishes from Falcon, and culture media and sera from GIBCO.

RESULTS

Stimulation of cGMP synthesis in normoxic cardiomyocytes. Stimulation of normoxic cardiomyocytes with 100 M SNAP or 1 M Uro in the presence of IBMX elicited a transient and rapid increase in the intracellular content of cGMP, with a maximum at 1 min (Fig. 1). In the extracellular medium, cGMP increased linearly with time during SNAP or Uro stimulation and reached ~30% of the intracellular concentration after 10 min. Cellular cGMP concentrations after 1 min of stimulation with SNAP or Uro were similar: 0.94 ± 0.06 and 1.12 ± 0.08 pmol/mg protein, respectively, vs. 0.04 ± 0.01 pmol/mg protein in nonstimulated cells. Responses to SNAP or Uro were not modified after 120 min of normoxic incubation: 96 ± 7 and 105 ± 15%, respectively, of preincubation values. The response to stimulation of cGMP synthesis with...
SNAP or Uro was significantly attenuated after a previous exposure to those agonists for 10 min (in the absence of IBMX) just before the 1 min of stimulation (49 ± 9 and 29 ± 15% of the responses to SNAP and Uro, respectively, in nonprestimulated cells, n = 3, P < 0.05).

Stimulation of sGC- and pGC-mediated cGMP synthesis was concentration dependent (Fig. 1B). The concentration of Uro necessary to reach half of the maximal effect (EC50) was 37 ± 22 nmol/l (n = 3). Maximal effects reached after stimulation of pGC with agonists selective for guanylyl cyclase type A receptors, Uro or ANP, were similar and lower than the effects reached after guanylyl cyclase type B receptor stimulation with CNP (Fig. 1, B and inset). In the following experiments, 100 μM SNAP and 1 μM Uro, ANP, or CNP were used to stimulate sGC and pGC, respectively.

Effect of simulated ischemia and reperfusion on cGMP synthesis. Simulated ischemia (hypoxia at pH 6.4) for 2 h decreases ATP content by 90% and intracellular pH to 6.42 (P < 0.05; Fig. 2) without increasing significantly LDH release with respect to normoxic incubation (14.5 ± 3.3 vs. 7.4 ± 1.2% of total LDH content, not significant). Simulated ischemia exerted a profound inhibitory effect on cGMP produced after stimulation with Uro (~50% of control after 120 min, P < 0.05; Fig. 2), ANP, or CNP (Table 1) but no significant effect on the response to SNAP (Fig. 2). Simulated reperfusion allowed complete and rapid recovery of Uro-mediated cGMP synthesis (Fig. 2).

To distinguish between low pH and ATP depletion effects found in simulated ischemia, cultures were sub-
Table 1. Effect of hypoxia and acidosis on ANP and CNP responses

<table>
<thead>
<tr>
<th>Condition</th>
<th>cGMP After Stimulation, % of response in normoxic cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ANP (1 μM)</td>
</tr>
<tr>
<td>Simulated ischemia</td>
<td>22.1 ± 8.2*</td>
</tr>
<tr>
<td>Acidosis</td>
<td>36.8 ± 2.3*</td>
</tr>
<tr>
<td>Hypoxia at pH 7.4</td>
<td>97.3 ± 9.4</td>
</tr>
</tbody>
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Values are means ± SE; n = 4. ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide. *P < 0.05.

Effect of extracellular acidosis. Cell incubation under normoxic conditions at pH 6.4 (acidosis) reduced the intracellular pH to 6.8 in 30 min (Fig. 3), whereas ATP content (Fig. 3) and LDH release (7.3 ± 1.5% of total cell content after 120 min) were not significantly modified. cGMP synthesis mediated by Uro decreased following a pattern very similar to that previously observed under simulated ischemia (Fig. 3). cGMP stimulation by ANP or CNP also diminished after 120 min of extracellular acidosis (Table 1). After restoration of extracellular pH, intracellular pH and the response to Uro were rapidly recovered. No changes were observed in sGC-dependent cGMP synthesis (Fig. 3).

Effect of hypoxia and metabolic inhibition at pH 7.4. Hypoxia without concomitant acidosis (pH 7.4) induced an even more profound ATP depletion than that observed during simulated ischemia (Fig. 4) without significantly altering intracellular pH (Fig. 4) or LDH release (12.2 ± 1.7%) but failed to reproduce the depressant effects of simulated ischemia on cGMP synthesis elicited by Uro (Fig. 4). Instead, a transient potentiation of cGMP synthesis in response to stimulation of pGC with Uro or sGC with SNAP was observed during the first 60 min of hypoxia. After 120 min of hypoxic incubation at pH 7.4, SNAP- and Uro-dependent cGMP synthesis returned to control levels. The response to ANP (a selective agonist of the pGC
SNAP, 1/H9262/ties was analyzed in cell fractions stimulated with 100 pH dependence of sGC and pGC activi-
cytic inhibition.

Contrary to hypoxia, no transient potentiation of Uro-
cell fractions obtained from nontreated cardiomyocytes de-
pH values (Fig. 5 A). sGC and pGC activities in cell
was significantly higher in cells preincubated for 120 min in a normoxic medium at pH 6.4 than in cells
activity could be detected at pH 6.4). However, regula-
acidosis (pH 6.4) during preincubation did not in
the central finding of the present study is that non-
substantially reduces pGC-dependent cGMP synthesis in
adult rat cardiomyocytes without significantly affect-
type A receptor) was also unaffected by hypoxia. On the
contrary, cGMP synthesis induced by CNP (a selective
agonist of the pGC type B receptor) was reduced by
>50% after 120 min of hypoxia in the absence of concomitant acidosis (Table 1). Simulated reoxy-
ation tended to decrease transiently cGMP synthesis
by guanylyl cyclase type B receptors, energy deple-
tion also played a significant role. A transient po-
tentiation of the two pathways of cGMP synthesis was
observed during hypoxia in the absence of extracellular
acids.

Throughout the study, we have assumed that the
intracellular accumulation of cGMP after 1 min of
stimulation with exogenous SNAP or Uro in the pres-
ence of IBMX reflects sGC- and pGC-mediated cGMP
synthesis, respectively. This assumption is supported
by 1) the minute cGMP content in the absence of
stimulation (<5% of cell content after stimulation of
sGC or pGC), 2) the almost total blockade of cGMP
degradation by 1 mM IBMX, and 3) the small contribu-
tion of cGMP efflux at 1 min of stimulation. This
assumption is, however, invalid during more prolonged
stimulation periods, because receptors rapidly desen-
sitize and egression of cGMP increases with time. Di-
ferences in the active transport of cGMP out of the cell
or in the desensitization process could explain differ-
ences in the time course of the response to stimulation
of cGMP synthesis, transient in this and other studies
(27) but continuous in others (12).

The effect of SNAP on cGMP synthesis in cardiomy-
ocyes is in agreement with previous studies using NO
donors (6). The results obtained with stimulation of
atriuretic receptors in these cells have been less con-
sistent. We were particularly interested in studying
how simulated ischemia altered stimulation of cGMP
synthesis by Uro, because this guanylyl cyclase type A
receptor agonist had been found to prevent hypercon-
tracture (11) and myocardial injury during reperfu-
sion (25) through stimulation of cGMP synthesis. How-
However, cardiomyocytes also express the guanylyl cyclase type
B receptor (17). Although some studies (4, 17) sug-
gested that this receptor was scarcely relevant in the
regulation of cGMP synthesis in cardiomyocytes, we, as
other authors (9, 24), observed synthesis of cGMP in
cardiomyocytes in response to stimulation of the gua-

FIG. 5. Guanylyl cyclase (GC) activity in cytosolic and particulate cell fractions. A: nontreated cardiomyocytes
were homogenized and centrifuged. Cell fractions were stimulated with 100 μM SNAP (soluble fraction), 1 μM Uro (par-
iculate fraction; type A receptors), or 0.1% Triton X-100 (particulate fraction; type A and B receptors) at different
pH values. B: soluble guanylyl cyclase (sGC) was isolated from cardiomyocytes previously preincubated for 120 min
at pH 6.4 or 7.4 and then assayed at pH 6.4 or 7.4. Values are means ± SE of 6 experiments expressed as percentage of
activity in the same conditions at pH 7.4 (22.1 ± 5.9 nmol cGMP·mg protein·min−1).
nlyl cyclase type B receptor with CNP. The origin of these discrepancies is not known, but because of the apparent relevance of guanylyl cyclase type B receptors in regulation of cGMP synthesis in our cells, CNP responses were also analyzed in the different conditions tested in the present study.

Few studies have analyzed the effects of ischemia on cGMP synthesis in myocardial tissue, and the results of these studies are discrepant. It has been reported that myocardial cGMP increases after 10–25 min of ischemia in the isolated rat heart (3, 18), whereas other studies in the same model found no change (20) or a reduction (23) after 30 min of hypoxia (23) or ischemia (20). Reduced myocardial cGMP content has also been reported in the in situ rat (28) and rabbit (10) heart after 30 min of transient ischemia. We recently reported a fall in the response to Uro in isolated rat heart subjected to 40 min of transient ischemia (11) and in pig myocardium subjected to 48 min of transient coronary occlusion (25). These discrepancies could be partially explained by a heterogeneous modulation depending on the cell type. In Fig. 6, we compared the effects of ATP depletion and intracellular pH on cGMP synthesis in cardiomyocytes and microvascular endothelial cells from rat heart. pGC-mediated cGMP synthesis in cardiomyocytes shows a pH dependence similar to that shown by cGMP synthesis evoked by sGC and pGC in endothelial cells, but the SNAP response in cardiomyocytes presents an unexpected resistance to intracellular acidosis. Also remarkable is the absence of effect of energy depletion in cardiomyocytes (with the exception of responses mediated by guanylyl cyclase type B receptors, as mentioned above), in contrast to the linear correlation observed in endothelial cells between ATP content and cGMP synthesis. To our knowledge, only one study has analyzed the effect of conditions simulating ischemia on the response to stimulation of cGMP synthesis in isolated cardiomycocytes (6). In agreement with our results, the authors of that study reported that a drop in the nucleotide triphosphate cell content (GTP) to <10% of the initial levels (after 60 min of hypoxia at pH 7.4) did not produce a decrease, but a substantial potentiation of cGMP synthesis, after stimulation with an NO donor.

This study demonstrates for the first time the profound depressant effect of acidosis on pGC-mediated cGMP synthesis in cardiomyocytes that accounted to a large extent for the depression of cGMP synthesis observed in this synthetic pathway under simulated ischemia (hypoxia plus acidosis). There was a good correlation between the time course of inhibition of the response to Uro and the time course of intracellular pH decrease during exposure to extracellular buffer at pH 6.4. Recovery of cGMP synthesis was rapid after normalization of the extracellular pH, indicating that acidosis did not induce changes in enzyme content or irreversible alterations of protein structure. The marked dependence of pGC activity in the particulate fraction of cardiomyocytes on pH is consistent with the hypothesis that the effect of acidosis is related to the pH dependence of the enzymatic reaction, as previously shown in endothelial cells (1). The activity of sGC was also decreased at acidic pH in cytosolic fractions, although to a lesser extent. The mechanism of the resistance of the SNAP response to intracellular acidosis in intact cardiomycocytes, in contrast to the susceptibility to low pH of sGC activity measured in cell fractions, is under investigation.

The absence of a general inhibitory effect of energy depletion on cGMP synthesis in cardiomyocytes is notable. Only the responses mediated by guanylyl cyclase type B receptors were found to be sensitive to energy depletion. However, although ATP concentration determines the concentration of GTP, the substrate for sGC and pGC, a direct correlation between ATP content and cGMP synthesis cannot be presumed. ATP depletion is associated with an increase of intracellular Mg2+ and inorganic phosphate concentrations (22, 26). Because the substrate of sGC and pGC is Mg2+-GTP and phosphate stimulates pGC (22), the effect of the increase in Mg2+ and phosphate on cGMP synthesis tends to antagonize the effects of ATP depletion. Moreover, stimulatory and inhibitory sites for ATP have been described in pGC type A and B receptors (5, 13, 19), and ATP depletion may also affect pGC activity through modifications in its phosphorylation state (19). Further studies are needed to discern which of these mechanisms is responsible for the absence of a general effect of the fall in ATP in cardiomyocytes, or even potentiation.

Fig. 6. cGMP synthesis elicited by sGC or particulate guanylyl cyclase stimulation vs. cellular ATP content or pH, for cardiomyocytes (CM) or microvascular endothelial cells (EC) from adult rat hearts. Cells were incubated under extracellular acidosis (circles; pH dependence), 120 min of hypoxia (diamonds), or different periods of metabolic inhibition with dinitrophenol (triangles; ATP dependence) and stimulated for 1 min with 1 μM Uro (filled symbols) or 100 μM SNAP (open symbols). These results have been partially presented in Figs. 3 and 4 and in Ref. 1.
In cardiomyocytes, cGMP has been found to modulate events that play an important role in the pathophysiology of injury secondary to ischemia-reperfusion. The present study demonstrates that, during ischemia, there is a reduced ability to synthesize cGMP through stimulation of pGC and that this effect is mainly mediated by the fall of intracellular pH. In these conditions, and presumably in any other situation causing an intracellular acidification, cardiomyocyte cGMP content would be strongly dependent on NO release. In addition, this study shows that the cGMP-signaling pathway can be differently modulated by ischemia in distinct myocardial cell types.

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